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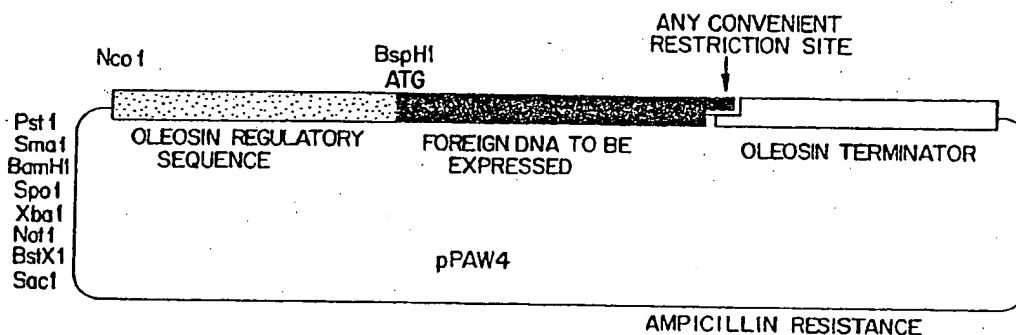
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(54) Title: OIL-BODY PROTEIN CIS-ELEMENTS AS REGULATORY SIGNALS



## (57) Abstract

DNA constructs comprising 5' untranslated sequences from genes active from the late globular stage through to embryo maturity are provided. These constructs may be used to obtain expression of a DNA sequence of interest during phases of embryogenesis which precede the accumulation of storage proteins.

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## OIL-BODY PROTEIN CIS-ELEMENTS AS REGULATORY SIGNALS

### INTRODUCTION

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#### Technical Field

This invention relates to upstream DNA sequences and their use to control expression of genes in developing plant seeds and their use.

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#### Background

Studies in plant gene expression have yielded a number of general conclusions concerning the elements that control expression. Plants, like other organisms both prokaryotic and eukaryotic, contain conserved or consensus sequences upstream (5') of the transcriptional start site of genes which appear capable of regulating transcriptional rates. In eukaryotes, these sequences include a motif found typically about 25 bp 5' to the transcriptional initiation site which has the sequence TATAA/TAA/T and is referred to as a TATA box. The role of this TATA box appears to be to define the transcriptional start for RNA polymerase II. A second upstream sequence is referred to as a CAAT box. Typically, this is found about 75 bases upstream of the transcriptional start and is associated with regulating the frequency of transcriptional initiation. In plants the consensus sequence may be either CCAAT or sometimes AGGA. However, neither of these alternative consensus sequences need be present in all plant genes. These sequence motifs and their DNA context within 70-90 bases upstream of the transcriptional start are often referred to as promoters. In general, 5' of the promoter region and most frequently within 2000 bases of it are cis-acting elements which confer a variety of properties on the promoter and which can modulate transcriptional activity in either a constitutive or a non-constitutive manner. These cis-acting sequences may be referred to as enhancers (if they are responsible for increases in transcription) or silencers (if they are responsible for

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decreases or suppression of transcription). Enhancers and silencers are frequently the sites at which nuclear proteins bind or interact. The modulating nuclear proteins are called trans-acting factors. They are considered to be very important for non-constitutive or regulated expression as they may be the major determinant of the activity of a gene in a particular tissue or organ or in response to an external stimulus. The relationship between this protein binding and the enhancer/silencer element may determine the transcriptional activity. The isolation of genes which are activated by heat, light or chemicals such as endogenous hormones or are activated in specific organs such as seeds, leaves or flowers has permitted analysis of factors which may determine how expression is regulated. In numerous, but not all, cases, it has been shown that the construction of chimeric genes which contain the promoter and optionally cis-elements from a given regulated gene and a coding sequence of a reporter protein not normally associated with that promoter gives rise to regulated expression of the reporter. The use of promoters from seed-specific genes for the expression of sequences in seed of genes that are either not normally expressed in a seed-specific manner or those that require an altered pattern of expression has been attempted on only a few occasions. In all cases to date, chimeric genes designed for seed-specific expression have used seed-storage protein regulatory signals and promoters. However, it is evident from work on storage protein gene expression that expression commences at a fairly late stage in embryogenesis, namely once the embryo has reached (in the case of dicots) the classical torpedo shape. Thus, although storage proteins express at high levels and their regulation is often transcriptional, the timing and level of expression may not be ideal for all seed-specific applications. It is, therefore, of interest to identify other seed-specific promoters and enhancers with temporal or cellular specificity different from that of seed storage proteins, such as those from oleosins.

#### Relevant literature

The following disclose organ or tissue-specific regulatory sequences used to produce tissue- or organ-specific expression in transformed plants. There are several by now "classical" examples of regulated gene expression in non-seed

protein chloramphenicol acetyl transferase could be expressed in a light-regulated and organ-specific manner in transgenic plants if the coding sequence for the reporter protein was fused with the promoter and upstream sequences from a pea gene encoding ribulose biphosphate carboxylase (Fluhr, Science (1986),  
5 232:1106-1112).

Sengupta-Gopalan et al. Proc. Natl. Acad. Sci. USA, (1985)  
82:3320-3324 reported expression of a major storage protein of french beans, called  $\beta$ -phaseolin, in tobacco plants. The gene expressed correctly in the seeds and only at very low levels elsewhere in the plant. However, the constructs used  
10 by Sengupta-Gopalan were not chimeric. The entire  $\beta$ -phaseolin gene including the native 5'-flanking sequences were used. Subsequent experiments with other species (Radke et al. (1988) Theor. App. Genet. 75:685-694) or other genes (Perez-Grau, L., Goldberg, R.B., 1989, Plant Cell, 1:1095-1109) showed the fidelity of expression in a seed-specific manner in both *Arabidopsis* and *Brassica*.  
15 Radke et al. (1988), vide supra, used a "tagged" gene i.e., one containing the entire napin gene plus a non-translated "tag".

In tissue and organ specific expression there have been several examples showing that sequences upstream of the transcriptional start may be used to confer tissue/organ specificity to a gene introduced into plants by genetic  
20 engineering. Examples include engineering seed-specific gene regulation (Radke et al. (1988) vide supra; Bustos et al. (1989), Plant Cell, 1:839-853). In both examples, sequences upstream of the coding sequences of seed proteins were linked to a reporter tag (either as RNA or protein) and seed specificity was conferred on expression of the reporter. These were all storage protein genes  
25 rather than oleosins. Seed storage proteins have different temporal expression patterns from oleosins.

The DNA motifs that might give rise to seed-specific expression are now the subject of many studies. Marcotte et al. (Marcotte, W.R., Russel, I.S.,  
30 Quantrano, R.S., 1989, Plant Cell, 1:969-976) studied the Em gene of wheat and proposed two motifs called "Em-boxes" which might be consensus sequences for seed-specific expression. Interestingly, one of these boxes called EM-2 is similar to that found in other storage protein genes from monocots (triticin-wheat) and

even dicots ( $\beta$ -conglycinin-soybean). Hatzopoulos et al. (1990, *Plant Cell*, 2:457-467) investigated the sequences directing embryo-specific expression of a carrot lipid-body protein gene. A number of AT rich motifs were identified, being protected from digestion during DNase treatment presumably by trans-acting proteins. The motifs identified, however, were not shown to be consensus motifs for other seed-specific genes.

DeClercq et al. *Plant Physiol.*, (1990), 94:970-979 used the promoter of the *Arabidopsis* 2S albumin and combined coding sequences from both the *Arabidopsis* and Brazil nut 2S albumins. Fusions were made in regions showing low conservation. Transformation of both tobacco and *Brassica napus* yielded seed-specific expression and correct accumulation of the modified storage proteins. Levels of expression were between 0.05% and 0.3% of total cellular protein.

Another example of this form of seed-specific expression of foreign sequences was the expression of leu-enkephalins in seeds. To obtain seed specific expression, a chimeric DNA sequence encoding a 2S albumin and a short oligonucleotide encoding leu-enkephalin (a pentapeptide) was included in the albumin coding sequences between the 6th and 7th cysteines of the native protein (Vanderkerhove et al. *Bio/Technology*, (1989) 7:929-932). Again this gene expressed in a seed-specific manner allowing the accumulation of up to 50 nmol leu-enkephalin per g of seed.

Genomic clones encoding oil-body proteins with their associated upstream regions have been reported for two species, maize (*Zea mays*, Bowman-Vance and Huang, (1987) *J. Biol. Chem.*, 262:11275-11279; and Qu and Huang, (1990) *J. Biol. Chem.*, 265:2238-2243) and carrot (Hatzopoulos et al. (1990) *Plant Cell*, 2:457-467). cDNAs and genomic clones have also been reported for one cultivated oilseed, *Brassica napus* (Murphy, et al. (1991), *Biochem. Biophys. Acta*, 1088:86-94; and Lee and Huang (1991) *Plant Physico* 96:1395-1397.) Reports on the expression of these oil-body protein genes in developing seeds have varied. In the case of *Zea mays*, transcription of genes encoding oil-body protein isoforms began quite early in seed development and were easily detected 18 days after pollination. In non-endospermic seeds such as the dicotyledonous plant



after pollination. In non-endospermic seeds such as the dicotyledonous plant *Brassica napus* (Canola), expression of oil-body protein genes seems to occur much later in seed development (Murphy, et al. (1989), Biochem. J., 258:285-293) than with corn.

### SUMMARY OF THE INVENTION

Methods and compositions are described for the exploitation of an oil-body protein transcriptional regulatory sequence and optionally its accompanying 5' untranslated leader sequence for the expression of heterologous genes in a seed-specific manner. The method includes the steps of transforming a plant cell with a DNA construct comprising the regulatory sequence and a DNA sequence other than the open reading frame native to the regulatory sequence, generating a plant from the transformed cell and growing it under conditions whereby seed is produced and the DNA sequence is expressed under the transcriptional control of the regulatory region. These sequences will be valuable in applications where expression of a seed-borne product needs to be modified, enhanced or suppressed. They could also be used to produce modified seeds containing foreign proteins to increase the intrinsic value of the seed.

### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a schematic diagram of vector pPAW4 enclosing an oleosin regulatory sequence, an initiation codon, foreign DNA to be expressed, an oleosin terminator sequence and an ampicillin resistance gene.

Fig. 2A shows the DNA sequence of an *Arabidopsis* genomic clone encoding a 18KDa oil-body protein. The open reading frame is interrupted by a short intron (which is marked) and the two exons are translated and indicated in IUPAC single letter amino-acid codes.

Fig. 2B shows the restriction fragment from an *Agrobacterium* EMBL3 genomic library which encloses the *Arabidopsis* 18KDa oil-body protein coding sequence. The approximate position of the coding region is highlighted.

Fig. 3 shows the effects of 10 $\mu$ M ABA on the developmental expression of oleosin mRNA using a Northern blot analysis of total RNA. A)

(70 $\mu$ g per lane) using 50 ng  $^{32}$ P dCTP labelled OB990 as a probe (spec. act.  $10^9$  dpm/ $\mu$ g DNA). Heart-(H): (13-day), torpedo-(T) (17-day), and cotyledonary-(C) (21-25 day) stage microspore-derived embryos with (+) and without (-) treatment for 48 h with 10 $\mu$ M ABA. The blot was exposed to Kodak XAR5 film at 70°C for 20 minutes. The apparent size difference of the mRNAs in the different lanes is due to interfering quantities of starch in the different mRNA preparations. All the lanes were equally loaded as judged by OD260 measurements and EtBr-staining. B) A 4.5 hour exposure of Fig. 3-A C) Relative intensity of the mRNA accumulation as determined by scanning densitometry.

Fig. 4 shows the tissue specificity of oleosin. 50  $\mu$ g of poly (A)+ RNA of roots (R), callus (Ca), Cotyledons (Co), leaves (L), and 24-day post-anthesis zygotic embryos (E) was probed with 50 ng of  $^{32}$ P dCTP labelled OB990 (spec. act.  $10^8$  dpm/ $\mu$ g DNA).

Fig. 5 shows the developmental sensitivity of oil body protein synthesis to applied ABA. An estimated 10,000 dpm were loaded per well for paired samples of controls (lanes A,C,E,G) and ABA-treated (lanes B,D,F,H). All samples were treated for 2d with ABA, then labeled for 4 h with 1.85 MBq/mL [ $^{35}$ S]methionine. Lanes A and B, 10-d-old cultures, sieved on 62 $\mu$ m screens to obtain globular embryos. Lanes C and D, 13-d-old cultures sieved on 125 $\mu$ m screens to obtain heart stage embryos. Lanes E and F, 17-d-old cultures, sieved on 250 $\mu$ m screens to obtain torpedo to early cotyledonary embryos. Lanes G and H, 25-d-old cultures, sieved on 500 $\mu$ m screens to obtain cotyledonary stage embryos.

#### BRIEF DESCRIPTION OF THE SPECIFIC EMBODIMENTS

In accordance with the subject invention, DNA constructs are provided which allow for modulation of plant phenotype in seed, particularly during early phases of embryogenesis. The DNA constructs provide for regulation of transcription in seed, using 5' untranslated sequences from genes active from the late globular stage through to embryo maturity (cotyledonary stage). Downstream from and under transcriptional initiation regulation of an oil body protein gene initiation region will be a DNA sequence of interest which will

be prepared which allow for integration of the transcription cassette into the genome of a plant cell. Conveniently, a multiple cloning site downstream from the seed specific transcriptional initiation region may be included so that the integration construct may be employed for a variety of DNA sequences in an efficient manner.

Of particular interest is a regulatory sequence from an oil body protein gene, preferably an oil body protein gene expressed in dicotyledonous oil seeds. It has been reported that oil-body proteins accumulate considerably later than either oils (triacylglycerides) or storage proteins. This later expression would limit the value of any promoters associated with these genes for seed-specific expression as they could not be used for modification of expression of genes during early phases of embryogenesis. Surprisingly, however, expression of these genes in dicotyledonous oilseeds was found to occur much earlier than had hitherto been believed. Thus, the promoters and upstream elements of these genes are valuable for a variety of uses involving the modification of metabolism during phases of embryogenesis which precede the accumulation of storage proteins.

Oil-body proteins have been identified in a wide range of taxonomically diverse species (see, for example, Moreau et al. *Plant Physiol.* (1980), 65:1176-1180; Qu et al. *Biochem. J.*, (1986) 235:57-65). These proteins are uniquely localized in oil-bodies and are not found in organelles of vegetative tissues. In *Brassica napus* (rapeseed) there are at least three polypeptides associated with the oil-bodies of developing seeds (Taylor et al. (1990), *Planta*, 181:18-26). The numbers and sizes of oil-body associated proteins may vary from species to species. In corn, for example, there are four immunologically distinct polypeptides found in oil-bodies (Bowman-Vance and Huang, 1988, *J. Biol. Chem.*, 263:1476-1481). Oleosins have been shown to comprise regions of alternate hydrophilicity, hydrophobicity and hydrophilicity (Bowman-Vance and Huang, 1987, *J. Biol. Chem.*, 262:11275-11279). The amino acid sequences of oleosins from corn, rapeseed and carrot have been obtained. See Qu and Huang, 1990, *J. Biol. Chem.*, 265:2238-2243, Hatzopoulos et al. 1990, *Plant Cell*, 2:457-467, respectively. In an oilseed such as rapeseed, oleosin may comprise between 8% (Taylor et al. 1990, *Planta*, 181:18-26) and 20% (Murphy et al. 1989,

Biochem. J., 258:285-293) of total seed protein. Such a level is comparable to that found for many seed storage proteins.

Of particular interest is a transcriptional initiation region associated with early embryogenesis, particularly the period preceding expression of storage proteins, so that in the early development of seed, it provides the desired level of transcription of the DNA sequence of interest. Normal plant embryogenesis typically goes through a series of defined phases. For dicotyledonous seeds, embryogenesis includes the following phases: globular stage, heart stage, torpedo stage, and cotyledonary stage. For the purposes of this application, the definition of these terms is provided by Ray, Steves, and Fultz in Botany, (Saunders College Publishing), Chapter 17, page 294. Normally, the transcriptional initiation region will be obtainable from a gene which is expressed in the early formation of seed. Desirably the transcriptional initiation region maintains its activity from the late globular through cotyledonary stage, more desirably continues active from the globular stage through the heart, torpedo and cotyledonary stages of embryogenesis. By obtainable is intended a transcriptional initiation region having a nucleotide sequence sufficiently similar to that of a natural oil body protein gene transcriptional initiation region sequence to provide for transcription in the early formation of seed. The sequence may be naturally occurring, synthetic or partially synthetic.

The transcriptional initiation region from the oil body protein generally will be provided in a cassette which will include in the 5'-3' direction of transcription, a transcriptional initiation region, a DNA sequence of interest and a transcriptional termination region, wherein the transcriptional regulatory regions are operably joined and functional in plant cells. One or more introns may also be present. After each manipulation, a DNA to be used in the final construct may be restricted and operably joined to other DNA to be used in the final construct, where each of the partial constructs may be cloned in the same or different plasmids. In a preferred embodiment, a coding sequence with a compatible restriction site may be ligated at the position corresponding to codon #1 of the oil-body protein gene. A schematic diagram of this substitution is shown in figure 1. The recombinant coding sequence may be inserted in such a way that it completely

replaces the coding sequence of the oil-body protein gene and is thus flanked at its 3' end by the oil-body protein gene terminator and polyadenylation signal.

Alternatively, polymerase chain reaction amplification may be carried out to produce DNA fragments containing the transcriptional initiation region conveniently flanked by restriction sites. The amplified fragments can be joined to the coding sequence for a polypeptide of interest, in a transcriptional or translational fusion, for example, to produce a chimeric gene in which the coding sequence of the polypeptide of interest is transcribed under the control of the transcription initiation region on the PCR amplified fragment.

The transcriptional initiation region may be native to or homologous to the host cell, or foreign or heterologous to the host cell. By foreign is intended that the transcriptional initiation region is not found in the wild-type host into which the construct comprising the transcriptional initiation region is inserted. Generally, the regulatory sequence comprises DNA of up to 1.5 Kb 5' of the translational start of an oil-body protein gene. This sequence may be modified at the position corresponding to the first codon of the desired protein by site-directed mutagenesis (Kunkel TA, 1985, Proc. Natl. Acad. Sci. USA, 82:488-492) or by introduction of a convenient linker oligonucleotide by ligation if a suitable restriction site is found near the N-terminal codon.

In some cases it will be desirable to express the DNA sequence of interest as a fusion protein, particularly as a fusion protein with the oil body protein. The DNA sequence of interest can be inserted by routine techniques into the oil body protein coding sequence, in frame with the oil body protein coding sequence, such that transcription of the chimeric gene will produce a fusion protein. The fusion protein will preferably contain the coding region for amino acids number 44 through 122 in the *Arabidopsis* oil body protein as shown in Figure 2A, or the equivalent region from an oil body protein of a species other than *Arabidopsis*, to provide for transport to the oil body in cases where this is desirable.

In order to isolate oil body protein coding sequences from other species, at least two approaches may be used. The first is to use the *Arabidopsis* clone described in the Examples as a probe in genomic libraries of other plant

species. This clone will hybridize well with oleosin clones from closely related species, in particular, essentially all cruciferous plants. For species which are evolutionarily divergent from *Arabidopsis*, for example, solanaceae, leguminaceae and all monocotyledons, an alternative method involves the use of an antibody raised against the gene product of an oleosin clone such as the *Arabidopsis* clone. This antibody may be used to screen a seed-derived cDNA expression library, for example using lambda gt11; Huynh et al. (1985) in cDNA Cloning, Vol. 1, A Practical Approach, Ed. Grover IRL Press, pp. 49-78. This approach yields a cDNA clone of the oleosin for the new species which may then be used to isolate the genomic clone from a genomic library of that species by standard DNA hybridization techniques.

The DNA sequence of interest may be any open reading frame encoding a peptide of interest, for example, an enzyme, or a sequence complementary to a genomic sequence, where the genomic sequence may be at least one of an open reading frame, an intron, a non-coding leader sequence, or any other sequence where the complementary sequence will inhibit transcription, messenger RNA processing, for example splicing or translation. The DNA sequence of interest may be synthetic, naturally derived or a combination thereof. Depending upon the nature of the DNA sequence of interest, it may be desirable to synthesize the sequence with plant preferred codons. The plant preferred codons may be determined from the codons of highest frequency in the proteins expressed in the largest amount in the particular plant species of interest.

The DNA sequence of interest may encode any of a variety of recombinant proteins. Examples of recombinant proteins which might be expressed by this procedure include anticoagulants, such as Hirudin, lymphokines such as those of the interleukin family, peptide hormones such as gonadotrophin releasing hormone, immunological reagents such as multi or single-chain antibodies and a variety of industrial valuable enzymes such as proteases, lipases and polyglucan hydrolases.

The termination region which is employed will be primarily one of convenience, since the termination regions appear to be relatively interchangeable. The termination region may be native with the DNA sequence of interest, or may

be derived from another source. Convenient termination regions are available and include the 3' end of the oil body protein gene terminator and polyadenylation signal from the same gene from which the 5' regulatory region is obtained.

Alternatively, a different terminator and polyadenylation signal may be employed with similar results, for example, the terminator of the nopaline synthase gene of *Agrobacterium*.

The expression cassette may additionally contain a means for identifying transformed cells and/or selecting for transformed cells. For example the recombinant gene may be linked with a constitutively expressed selectable marker such as a gene for antibiotic resistance or herbicide resistance or a screenable marker, such as a gene conferring bioluminescence or colored properties to transformed cells.

The DNA sequence of interest flanked at its 5' end by the oil-body protein promoter and regulatory sequences and at its 3' end by a terminator may be introduced into a suitable transformation vector including *Agrobacterium* Ti or binary plasmids, or a simple cloning plasmid (e.g., pUC19, pBR322) for use in direct DNA uptake to plant cells via microinjection, electroporation, PEG-mediated uptake or a biolistic method. These methods are well known to those skilled in the art of plant transformation. See, for example, Horsch et al. (1985), *Science*, 227:1229-1231; Newhaus and Spangenberg (1990). *Physiol. Plant*, 79:213-217; and Sandford et al. (1990), *Physiol. Plant*, 79:206-209.

Transformed plants may be obtained from the transformed cells using standard regeneration protocols (see for example: Moloney et al. (1989), *Plant Cell Rep.*, 8:238-242) compatible with the transformation method.

The expression cassette, constructed as described above, expresses essentially preferentially in developing seeds. The plant cells which have been transformed with an appropriate fusion peptide therefore are grown into plants in accordance with conventional ways and allowed to set seed. See, for example, McCormick et al., *Plant Cell Rep.* (1986) 5:81-84. Two or more generations may be grown and either pollinated with the same transformed strain or different strains, identifying the resulting hybrid having the desired phenotypic characteristic, to ensure that the subject phenotypic characteristic is stably

maintained and inherited and then seeds harvested for isolation of the peptide of interest or for use to provide seeds with the new phenotypic property. The regenerated plants are then cultivated identically to non-recombinant plants in growth chambers, greenhouses or in the field and will show seed-specific expression of the recombinant gene at the mRNA level and often at the level of polypeptide or protein.

It is possible that the polypeptide/protein will itself be valuable and could be extracted and, if desired, further purified. Alternatively the polypeptide/protein or even the mRNA itself may be used to confer a new biochemical phenotype upon the developing seed. New phenotypes could include such modifications as altered seed-protein or seed oil composition, enhanced production of pre-existing desirable products or properties and the reduction or even suppression of an undesirable gene product using antisense, ribozyme or co-suppression technologies (Izant and Weintraub (1984), Cell 36: 1007-1015, antisense; Hazelhoff and Gerlach (1988), Nature 334:585-591, ribozyme; Napoli, et al. (1990), Plant Cell, 2:279-289, co-suppression).

If the transformation has been performed to produce a new seed protein or peptide which requires extraction, this can be done using aqueous extraction with or without low concentrations of detergents, such as non-denaturing amounts of sodium dodecyl sulphate (SDS), Triton-X-100, Tween 20, MEGA-8 or any other detergent known not to irreversibly inactivate the desired protein. To extract the protein or polypeptide, dry seeds are ground by hand or in a mechanical grinder to produce an aqueous slurry or suspension. This can be resolved into three phases (particulate, aqueous soluble, and hydrophobic) by centrifugation, such as at 50,000 x g. Depending upon the nature of the product, it may be further purified in each of these phases and after solubilization, may be selectively precipitated by the use of ammonium sulfate or purified using column chromatography, for example, using ion exchange, gel filtrates or affinity matrices.

While the ideal host for the regulatory sequence reported here would be a cruciferous plant, it is possible to use these promoters in a wide variety of plant species given the relatively high conservation oleosin of genes. The major



barrier to the use of these promoters is between monocotyledonous and dicotyledonous species. For transformations involving this specific expression on a monocot, a monocot oleosin regulatory sequence should be used. For dicot seed-specific expression, a dicot oleosin regulatory sequence should be employed. The reported sequence can be used in a wide variety of dicotyledonous plants, including all members of the *Brassica* genus and crucifers in general. Solanaceous plants, such as tobacco and tomato, also recognize the sequences and show correct regulation of expression in developing seeds.

It is expected that the desired proteins would be expressed in all embryonic tissue, although different cellular expression can be detected in different tissues of the embryonic axis and cotyledons. This invention has a variety of uses which include improving the intrinsic value of plant seeds by their accumulation of altered polypeptides or novel recombinant peptides or by the incorporation or elimination of a metabolic step. In its simplest embodiment, use of this invention may result in improved protein quality (for example, increased concentrations of essential or rare amino acids), improved liquid quality by a modification of fatty acid composition, or improved or elevated carbohydrate composition. Examples include the expression of sulfur-rich proteins, such as those found in lupins or brazil nuts in a seed deficient in sulphurous amino acid residues. Alternatively, a fatty acyl coenzyme A (COA) a transferase enzyme capable of modifying fatty acid ratios in triglycerides (storage lipid) could be expressed. In cases where a recombinant protein is allowed to accumulate in the seed, the protein could also be a peptide which has pharmaceutical, industrial or nutritional value. In this case, the peptide could be extracted from the seed and used in crude or purified form, as appropriate for the intended use. The protein could be one truly foreign to the plant kingdom, such as an animal hormone, enzyme, lymphokine, anticoagulant, or the like could be expressed in seed. The heterologous protein could then be extracted from the seeds and used for experimental, nutritional or pharmaceutical purposes after partial or complete purification.

The following examples are offered by way of illustration and not by limitation.

EXAMPLESExample 1

The oil body protein gene from *Arabidopsis* was isolated on a 15kb insert present in a clone from an *Arabidopsis thaliana* v. Columbia genomic library in phage  $\lambda$  EMBL3A by hybridization to a *B. napus* oleosin clone. A 1.8kb fragment containing approximately 868 base pairs 5' of the oleosin protein translational start was subcloned into a plasmid vector. The *Arabidopsis* 18 KDa oleosin gene is conveniently cloned as a 1803 bp fragment flanked by NcoI and KpnI sites in a vector called pPAW4 (see Figure 1). In order to convert the fragment into an expression cassette for general use with a variety of foreign/alternative genes, two modifications must be made. Firstly, using the technique of site-directed mutagenesis (Kunkel, supra) mutations at positions -2, -1 and +4 are introduced using a mis-matched oligonucleotide. The mutations required are A to T (-2), A to C (-1) and G to A (+4). These mutations have the effect of creating a BspHI site at positions -2 to +4. The BspHI site (T/CATGA) encloses the ATG initiation codon and gives a recessed end compatible with an NcoI cut. A second modification involves digestion with EcoRV and MscI which releases a 658 bp fragment containing most of the coding sequence of the native oleosin. This leaves blunt ends at the cut sites which on separation of the vector and an ancillary sequence from the EcoRV-MscI fragment, permits recircularization of the vector-promoter-terminator combination. This recircularization is performed in the presence of an oligonucleotide linker containing restriction sites not found in the original 1803 Kb fragment.

On recircularization, a plasmid containing all the upstream sequences of the oleosin gene, a transcriptional start site and an initiation codon embedded in a BspHI site is obtained. Thirty-one bases downstream of this is a short polylinker containing one or more unique restriction sites. To introduce any DNA sequence into this cassette the foreign sequence should have, or should be modified to contain, a BspHI or NcoI site at the initial ATG position. For sequences to be expressed as proteins this will assure conservation of the distance between the "cap" site and the initiator codon.

The DNA sequence to be inserted should terminate with a cohesive end of a restriction site not found on the plasmid. The polylinker interposed into the expression cassette may be chosen with this site in mind. Digesting the plasmid with BspH1 and the appropriate restriction enzyme for the 3' end of the foreign sequence will ensure that a directional cloning of the desired DNA fragment may be effected. Using appropriate ligation conditions, the plasmid expression cassette with BspH1 and a site compatible with the desired DNA fragment are incubated together to produce a ligated product as shown in Figure 1.

The complete construct from Nco1-Kpn1 is now excised and introduced into an appropriate plant transformation vector such as an *Agrobacterium* plasmid. In order to introduce the construct into common *Agrobacterium* plasmids such as Bin 19 (Bevan, Nucl. Acid Research (1984) 12:8711-8721) it may be necessary to use one of the additional restriction sites in plasmid pPAW4. In one scenario the plasmid could be cut with Sma1 and Kpn1. The resulting purified fragment then is ligated to a Kpn1 oligonucleotide linker and digested with Kpn1. This provides a non-directional Kpn1 fragment for introduction into Bin 19. Alternatively, the construct may be excised with Kpn1 and BamH1 and ligated directionally into pBIN 19 previously cut with the same restriction enzymes. The resulting *Agrobacterium* binary plasmid is mobilized into a disarmed *Agrobacterium* strain by tripartite mating (Ditta, et al. (1980), PNAS 77: 7347-7351) or DNA transformation of competent *Agrobacterium* (An, (1988), Plant Mol. Biology Manual, A3 1-19, Kluwer Academic, Dordrecht, Netherlands).

The *Agrobacterium* harboring the recombinant Bin 19 is used to transform any susceptible plant, e.g., *Brassica* sp. by standard explant co-cultivation (Horsch et al. (1985), supra). The transformed cells are selected in culture with kanamycin using the co-transferred antibiotic resistance genes (neomycin phosphotransferase) also contained between the T-DNA borders of pBin 19. These transformed cells are induced to regenerate whole plants by standard procedures (e.g. for an oilseed such as rapeseed. See, Moloney et al. Plant Cell Rep., (1989), 8: 238-242). The regenerated plants are permitted to flower and are self-fertilized (or may be cross-fertilized). In cases where the foreign DNA in the construct encodes a translatable product, this product may be isolated from

aqueous extractions of the mature seed and subsequent fractionation of the slurry by centrifugation (30 min at 100,000 xg). Depending on the desired product it may partition with any one of the three phases obtained. It may be localized in the pellet, aqueous soluble phase or in the lipid film on the surface of the centrifuged sample.

Alternatively, it may not be necessary to extract the product as the purpose of the expression may be to divert metabolism in the seed thus changing the phenotype of the seed (e.g. by altering size or colour of the seed, changing the ratio of fatty acid residues in the seed or interdicting a particular metabolic step considered to render the seed less useful or valuable. Such metabolic steps might include the production of antinutritional secondary products which reduce the value or desirability of the seed when present. In such cases, the seed, per se, is simply harvested and used in accordance with usual procedures.

## Example 2

A number of constructs containing varying amounts of the DNA sequence from the 5' transcriptional initiation region of the *Arabidopsis* oleosin gene joined operably to the coding region for  $\beta$ -glucuronidase (GUS) were prepared using PCR. The constructs are designated according to the amount of the oleosin 5' region contained, for example, the 2500 construct has approximately 2500 base pairs of the oleosin 5' region. The constructs were introduced into *Brassica napus* and tobacco and the expression of the  $\beta$ -glucuronidase gene was measured as described in detail below. The GUS expression results of five constructs, the 2500, the 1200, the 800, the 600 and the 200 constructs in transformed *Brassica napus* plants are shown in Table I. A negative control (untransformed plant) is also shown. The GUS expression results of two constructs, the 2500 and the 800 constructs, in transformed tobacco plants are shown in Table II. Table III shows the developmental timing of the expression of the oleosin promoter in transgenic embryos.

The constructs were made using standard molecular biology techniques, including restriction enzyme digestion, ligation and polymerase chain

reaction (PCR). As an illustration of the techniques employed, the construction of the 800 construct is described in detail.

In order to obtain a DNA fragment containing approximately 800 base pairs from the 5' transcriptional initiation region of the *Arabidopsis* oleosin gene in a configuration suitable for ligation to a GUS coding sequence, a PCR based approach was used. This involves the use of the polymerase chain reaction to amplify the precise sequence desired for the expression analysis. To perform the necessary PCR amplification, two oligonucleotide primers were synthesized (Milligen-Bioscience, Cyclone DNA synthesizer) having the following sequences:

Pst 1 *oleosin seq*

5' primer: 5'CACTGCAGGAACTCTCTGGTAA 3'  
(GVR10)

The italicized bases correspond to nucleotide positions -833 to -817 in the sequence reported in Fig. 2A. The additional nucleotides 5' of this sequence in the primer are not identical to the oleosin gene, but were included in order to place a Pst1 site at the 5' end of the amplification product. The Pst1 site is underlined.

A second (3') primer was synthesized which had the following sequence:

3' primer (ALP 1)

BamH1 *oleosin seq*

5-CTACCCGGGATCCTGTTTACTAGAGAGAATG-3

Sma1

This primer contains the precise complement (shown in italics) to the sequence reported in Fig. 2A from base -13 to -30. In addition, it contains a further 13 bases at the 5' end. This sequence is not complementary to the oleosin gene, but was added to provide two (overlapping) restriction sites, Sma1 and BamH1, at the 3' end of the amplification product to facilitate cloning of the PCR fragment.

These two primers were used in a PCR amplification reaction to produce DNA fragment containing the sequence between nucleotides -833 and -13 of the oleosin gene with a PstI site at the 5' end and SmaI and BamHI sites at the 3' end. PCR amplification was performed using the enzyme Taq polymerase (Perkin-Elmer-Cetus) using the conditions recommended by the enzyme manufacturer and a temperature program of 92°C (denaturation) 1 min, 55°C (annealing) 1 min and 72°C (elongation) 1 min. The template was the oleosin genomic clone shown in Figure 2B, top panel, which in the original  $\lambda$  library isolate contained approximately 15 kilobases of *Arabidopsis* DNA.

The amplification product (OLEO p800) was gel purified on 0.7% agarose, recovered using the glass bead method of Vogelstein and Gillespie (Preparative and analytical purification of DNA from agarose. Proc. Natl. Acad. Sci. USA 1979 76:615-619) and digested with PstI. The digestion product was gel purified and end filled using DNA polymerase Klenow fragment then cut with SmaI to produce a blunt ended fragment. This was cloned into the SmaI site of pUC19 to yield the plasmid pUC OLEOp800. Using the asymmetric positioning of the AccI site in the insert (at the position corresponding to -649 in the oleosin gene as shown in Figure 2B) it was possible to select both orientations of insertion into pUC vector. The clone having the insert oriented such that the 5' most end of the amplified fragment (in the direction of transcription) is proximal to the unique Hind III site in the pUC19 cloning vector and the 3' most end of the amplified fragment is proximal to the unique Eco RI site in the pUC19 closing vector.

The resulting plasmid was then cut with BamHI to yield the fragment OLEOp800 flanked by BamHI sites. This fragment, BamHI-OLEOp800, was cloned into the BamHI sites of a BamHI digested plasmid designated HspGUS1559. HspGUS1559 is a plasmid used as a binary vector in *Agrobacterium*, derived from the vector pCGN 1559 (MacBride and Summerfeldt, 1990, Plant Molecular Biology, 14, 269-276) with an insert containing heat shock promoter (flanked by BamHI sites), the  $\beta$ -glucuronidase open reading frame and a nopaline synthase terminator (derived from pBI221, Jefferson RA in Cloning Vectors 1988, Eds. Pouwels P., Enger-Valk BE, Brammer WJ., Elsevier Science

Pub BV, Amsterdam section VII, Ai11). BamH1 digestion of HspGUS1559 results in the release of the heat shock promoter and permits the insertion of any other BamH1 fragment in its place. The BamH1-OLEOp800 fragment was ligated into this site to yield the *Agrobacterium* pOLEOp800GUS1559. This plasmid was used to transform *E. coli* and the amplified plasmid was introduced into *Agrobacterium* (strain EHA101) by electroporation as described above (Rogers et al., 1988, Plant Molecular Biology Manual, A2: 1-12, Eds. Gelvin S. and Schilperoort, R. Kluwer Academic, Dordrecht, Netherlands).

The resultant *Agrobacterium* strain (EHA 101 x pOLEOp800GUS1559) was used to transform *Brassica napus* plants by the method of Moloney et al. (Moloney, M.M., Walker, J.M., Sharma, K.K. (1989) Plant Cell Reports 8:238-242) or tobacco plants by the method of Horsch et al. (Horsch et al. Science (1985) 227:1299-1302). The resultant transgenic plants were allowed to set seed, and GUS expression assays (Jefferson R.A. (1987), Plant Mol. Biol. Rep. 5 387-405) were performed on the developing seeds and also on non-reproductive plant parts as controls. GUS expression reported is an average obtained from approximately five seeds from each of approximately five different transgenic plants.

The other constructs were prepared by the same PCR method described above using the appropriate primers for amplifying the -2500 fragment, the -1200 fragment, the -600 fragment or the -200 fragment. The results in *Brassica napus* expressed as specific activity of GUS enzyme are shown in Table I. The results in tobacco are shown in Table II.

These results demonstrate that the oleosin fragment from -833 to -813 used in the 800 construct contains sufficient information to direct seed-specific expression of a reporter gene in transgenic *Brassica napus* embryos as early as heart stage and that the *Arabidopsis* oleosin promoter is capable of directing transcription in plants other than *Arabidopsis*. These experiments also show that the sequences present in this promoter construct contain the cis elements required for an increase in transcription in response to the addition of abscisic acid, a characteristic of the native oleosin promoter.

It should be noted that the seed-specific expression demonstrated here does not depend on interactions with the native terminator of an oleosin gene 3' end. In this example, the 3' oleosin terminator was replaced by a terminator derived from the nopaline synthase gene of *Agrobacterium*. Thus, the sequence in the 800 construct is sufficient to drive the desired expression profile independent of ancillary sequences.

5



Table I

Seed Specific Expression in Brassica napus

GUS Activity (in pmol product/min/mg protein)

Promoter/GUS Construct	Seed (Torpedo Stage)		Root	Leaf	Stem	Seed (Late-Cotyledonary)
	+ABA*	-ABA				
2500	10,185	7,709	444	46.9	88.2	11,607
1200	18,298	1,795				8,980
800	2,250	475	285	277	650	7,130
600	1,506	144				1,365
200	18.1	64.8	260	5.9	26	11
Negative Control-Non-transformed Plant	18.4	13.9	300	6.1	30	14

\*ABA is treatment for 24 hours with  $10^{-5}$ M abscisic acid prior to GUS activity measurement

Table II  
Seed Specific Expression in Tobacco

Promoter/GUS Constructs	GUS Activity (in pmol product/min/mg protein) Mature Seeds
2500	11,330
800	10,970

Table III  
Developmental Expression in *Brassica napus*

GUS ACTIVITY (in pmol product/min/mg protein)<sup>a</sup>

Promoter/ GUS <u>Construct</u>	Heart <u>Stage</u>	Torpedo <u>Stage</u>	Early Cotyledonary <u>Stage</u>	Mid- Cotyledonary <u>Stage</u>	Late Cotyledonary <u>Stage</u>
2500	272	1207	2541	1819	11,607
1200	124	262	388	5094	8,980
800	149	260	962	2617	7,128
600	59	41	29	38	1,365
200	30	25	15	20	11
Negative Control			11	14	14

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

5       The invention now being fully described, it will be apparent to one of ordinary skill in the art that many changes and modifications can be made thereto without departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

5 1. A method of expressing a DNA sequence of interest in a seed cell, said method comprising:

growing a plant capable of developing seed, wherein said plant comprises cells containing integrated into their genome an expression cassette comprising in the 5'-3' direction of transcription as operably linked components, a transcriptional regulatory region obtainable from an oil body protein gene, a DNA sequence of interest heterologous to said regulatory region, and a transcriptional termination region, under conditions whereby seed is produced in which said DNA sequence is expressed under transcriptional control of said regulatory region.

2. The method according to Claim 1, wherein said gene is expressed during a phase of embryogenesis which precedes accumulation of storage proteins.

15 3. The method according to Claim 2 wherein said phase is from the formation of a globular embryo through to early cotyledonary stage.

4. The method according to Claim 1, wherein said gene is from a dicotyledonous plant.

20 5. The method according to Claim 2, wherein said phase is selected from the group consisting of globular, heart, torpedo and cotyledonary.

6. The method according to Claim 4, wherein said dicotyledonous plant is *Brassica napus* or *Arabidopsis*.

7. The method according to Claim 1, wherein said plant is other than *Arabidopsis*.

25 8. A DNA construct comprising:  
a chimeric gene comprising a transcriptional regulatory region obtainable from a gene which encodes an oil body protein fused to a DNA sequence of interest heterologous to said regulatory region.

30 9. The DNA construct according to Claim 8, wherein said gene is obtainable from a member of *Brassicaceae*.

10. The DNA construct according to Claim 8, wherein said transcriptional regulatory region is obtainable from *Brassica napus* or *Arabidopsis*.

11. An expression cassette comprising:

as operably linked components, a transcriptional regulatory region obtainable from an oil body protein gene, a DNA sequence of interest heterologous to said regulatory region, and a transcriptional termination region.

12. An isolated transcriptional regulatory region obtainable from an oil body protein gene.

13. The isolated transcriptional regulatory region of Claim 12 wherein said gene is obtainable from *Brassica napus* or *Arabidopsis*.

14. A plant comprising:

cells containing integrated into their genome a chimeric gene comprising a transcriptional regulatory region obtainable from an oil body protein gene, wherein said transcriptional regulatory region is fused to a DNA sequence of interest heterologous to said regulatory region.

15. Seed comprising:

cells containing integrated into their genome, a chimeric gene comprising a transcriptional regulatory region obtainable from an oil body protein gene, wherein said transcriptional regulatory region is fused to a DNA sequence of interest heterologous to said regulatory region.

16. Seed according to Claim 15, wherein said transcriptional regulatory region is from an oil-body protein gene obtainable from *Brassica napus* or *Arabidopsis*.

17. Seed according to Claim 15, wherein said seed is dicotyledonous.

18. Seed according to Claim 16, wherein said seed is an oilseed.

19. A method for altering seed-specific metabolism, said method comprising:

growing a plant capable of developing seed, wherein said plant comprises cells containing integrated into their genome an expression cassette comprising in the 5'-3' direction of transcription, a transcriptional initiation region from an oil body protein gene, a DNA sequence of interest other than a sequence native to said initiation region, and a transcriptional termination region, under conditions whereby seed is produced in which said DNA sequence is expressed under transcriptional control of said transcriptional initiation region.

20. The method according to Claim 19, wherein said altering is reducing or suppressing expression of endogenous genes expressed in plant seeds.

21. The method according to Claim 19, wherein said transcriptional initiation region includes a silencer element.

5 22. The method according to Claim 19, wherein a transcribed strand of said DNA sequence is complementary to mRNA endogenous to said cells.

23. A method for producing novel polypeptides in seed, said method comprising:

10 growing a plant capable of developing seed, wherein said plant comprises cells containing integrated into their genome an expression cassette comprising in the 5'-3' direction of transcription, a transcriptional initiation region obtainable from an oil body protein gene, a DNA sequence of interest encoding a polypeptide foreign to said plant, and a transcriptional termination region, under conditions whereby seed is produced in which said DNA sequence is expressed under transcriptional control  
15 of said transcriptional initiation region.

24. A plant part, comprising a DNA construct according to Claim 8.

25. A plant part according to Claim 24, wherein said part is a leaf, stem, root, flower, fruit or seed.

26. An isolated DNA comprising:

20 a transcriptional regulatory region which provides for expression of a DNA sequence of interest during a phase of embryogenesis which precedes accumulation of storage proteins, wherein said regulatory region is obtainable from oil body protein.

27. The isolated DNA according to Claim 26, wherein the nucleotide  
25 sequence of said DNA comprises:

a nucleotide sequence from nucleotide -867 to nucleotide 1 of the genomic clone shown in Figure 2A.

28. A method for expressing a DNA sequence of interest in a host plant during a phase of embryogenesis which precedes accumulation of storage proteins,  
30 said method comprising:

transforming said host plant with a construct comprising a DNA sequence of interest operably fused to a transcriptional regulatory region, wherein said regulatory region is obtainable from an oil body protein gene; and

growing said plant under conditions whereby seed is produced and said DNA sequence of interest is expressed under transcriptional control of said regulatory region.

29. The method according to Claim 28 wherein said host is a dicotyledenous oil seed cell.

30. The method according to Claim 29, wherein said gene is obtainable from the group consisting of

- (a) *Brassica napus*;
- (b) *Zea mays*;
- (c) carrot; and
- (d) *Arabidopsis*.

31. A method for obtaining a purified polypeptide of interest, said method comprising:

transforming a host plant cell with a DNA construct under genomic integration conditions, wherein said DNA construct comprises a first DNA sequence encoding a polypeptide of interest inserted in reading frame into an oil body protein (OBP) gene which includes a sufficient portion of the regulatory region 5' to the translational start site of said OBP gene to provide for expression of said gene in seed, wherein said sequence is inserted at a site in said gene so that expression of said DNA sequence is controlled by said regulatory region, whereby said DNA construct becomes integrated into the genome of said plant cell;

growing said plant to produce seed whereby said polypeptide of interest is expressed as a fusion protein with the expression product of said OBP gene;

isolating oil bodies from the cells of said seed;

disrupting said oil bodies whereby said fusion protein is released; and

purifying said polypeptide of interest.

32. A method for obtaining a polypeptide of interest in an oil body, said method comprising:

expressing said polypeptide in seed as a fusion protein with an oleosin, or a sufficient portion thereof to provide targetting to said oil body.



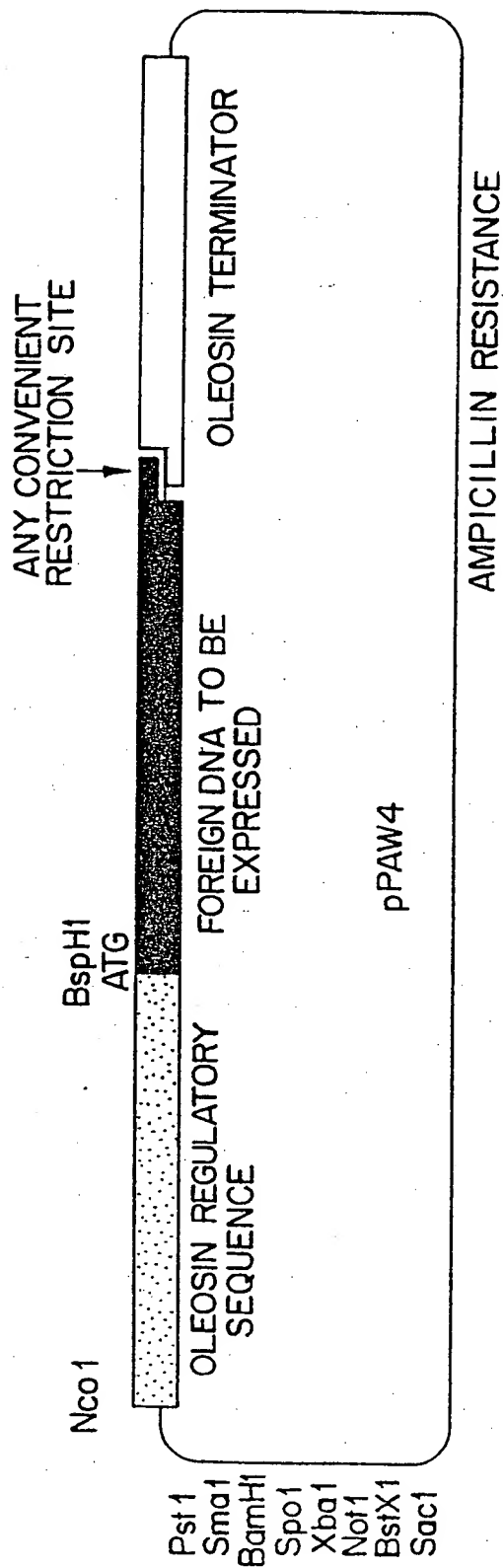


FIG. 1

NcoI  
-867 CCA<sup>31</sup>TGGGTATACCCAACTCGGCTTGGTCACACCAGGAAC<sup>31</sup>TCTCTGGTAAGCTAGCTCCACTGCCAGAAACAACCGGCGCCAAATTGC  
-777 CGGAATTGCTGACCTGAAGACGGAACATCATCGTCGGTCTTGGCGGATTGCGCGGAAGATGGGTCAAGCTTGGGCTTGAGGACGAGAC  
-687 CCGAATCGAGTCTGTTGAAAGGTGTTCAATTGGGATTTGTATACGGAGATTGGTCGTGAGAGGTTIGAGGGAAAGGACAAATGGGTTTG  
-597 GCTCTGGAGAAAGAGAGTGGCGCTTIIAGAGAGAGAAATTGAGAGGTTIIAGAGAGAGATGCGGCGCGATGACGGGAGGAGAGACGACGAGG  
-507 ACCTGCATTATCAAAGCAGTGACGTGGTGAAATTTGGAACTTTTAAGAGGCAGATAGATTTATTATTTGTATCCATTTTCTTCATTGTTTC  
-417 TAGAATGTCGCGGAACAAATTTTAAACTAAATCTCTAAATTTTCTAATTTTGTGCCAATAGTGGATATGTGGGCCGTATAGAAAGGAAT  
-327 CTATTGAAGGCCCAAACCCATACTGACGAGCCCCAAAGGTTTCGTTTTGCGTTTTATGTTTCGGTTCGATGCCAACGCCACATTCTGAGCTA  
-237 GGCAAAAACAAACGTGTCTTTGAAATAGACTCCTCTCGTTAA<sup>1</sup>CACATG<sup>1</sup>CAGCGGCTGCATGGTGACGCCATTAAACACGTGGCCTACAATT  
-147 GCATGATGTCTCCATTGACAGTGACTTCTCGTCTCCTTTCTTAATATCTAACAAACACTCCTACCTCTTCCAAAATATATACATC  
-57 TTTTGTATCAATCTCTCAAAATCTCATTCTCTCTAGTAAACAAGAACAAAAAATGGCGGATACAGCTAGAGGAACCCATCAGCAT  
34 ATCATCGGCAGAGACCGAGTACCCGATGGGCGGAGACCGAGACCAGTACCAAGATGTCCGGACGAGGATCTGACTACTCCAA<sup>1</sup>GTCTAGG  
I I G R D Q Y P M M G R D R D Q Y Q M S G R G S D Y S K S R  
T A D T A R G T H H D

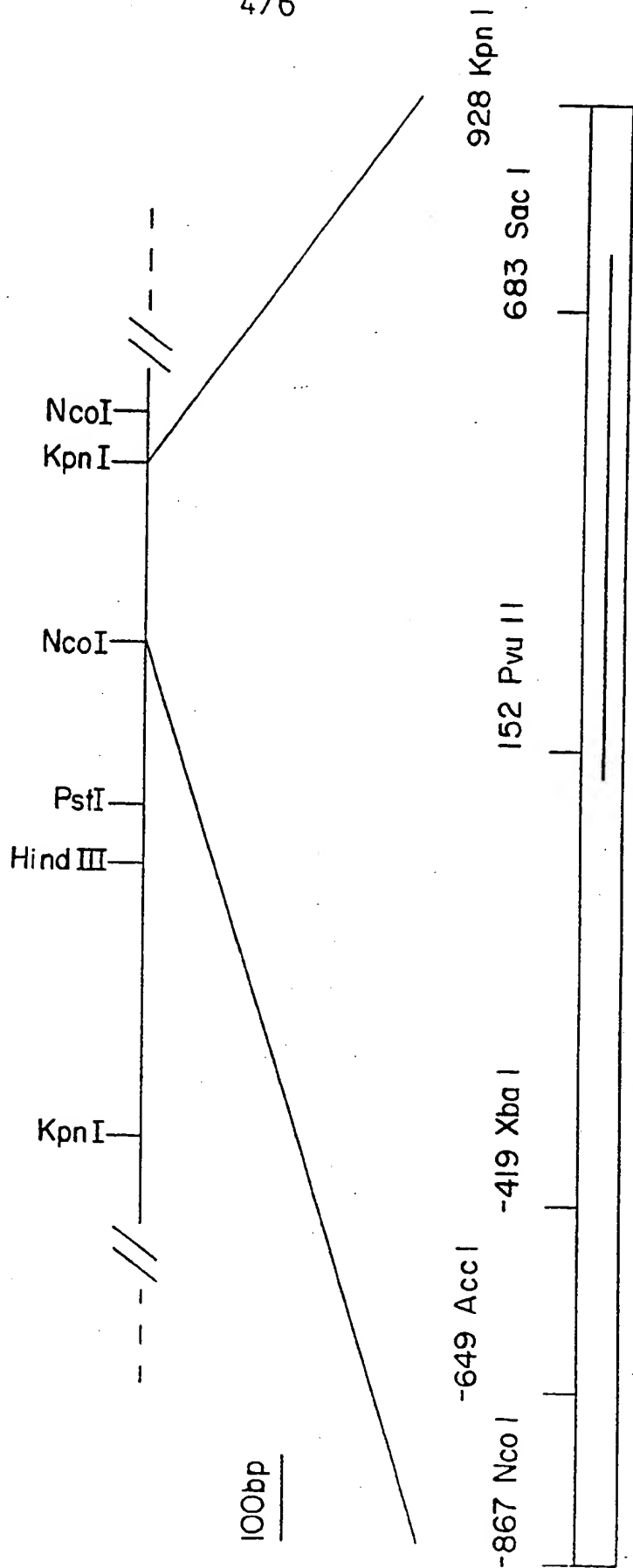
3/6

Q I A K A A T A V T A G G S L L V L S S L T L V G T V I A L  
 124 CAGATTGCTAAAGCTGCAACTGCTGTACAGCTGGTGGTTCCCTCCTTGTCTCTCCAGCCTTACCCCTTGTGGAACTGTGCTAGCTTTG  
 T V A T P L L V I F S P I L V P A L I T V A L L I T G F L S  
 214 ACTGTTGCAACACCTCTGCTGTTATCTTCAGCCCAATCCTTGTCCTCCGGCTCTCATCAGTTGCACCTCTCATCACCAGGTTTCTTTCC  
 S G G F G I A A I T V F S W I Y K .....  
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 664 GCTCAGGATCTGAAAGACAGAGCTCAGTACTACGGACAGCAACATACTGGTTGGGAACATGACCCGTGACCCGTACTCGTGGTGGCCAGCAC  
 T T \*  
 754 ACTACTTAAGTTACCCCACTGATGTCATCGTCATAGTCCAATAACTCCAATGTCGGGGAGTTAGTTTATGAGGAATAAAGTGTTTAGAAT  
 844 TTGATCAGGGGGAGATAATAAAAGCCGAGTTTGAATCTTTTTTGTATAAGTAATGTTTATGTGTGTTTCTATATGTTGTCAAATGGTACC<sup>KpnI</sup>

FIG. 2A-2

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1 kb



SUBSTITUTE SHEET

FIG. 3A

H      T      C  
-   +   -   +   -   +

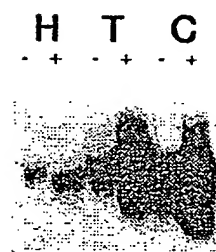
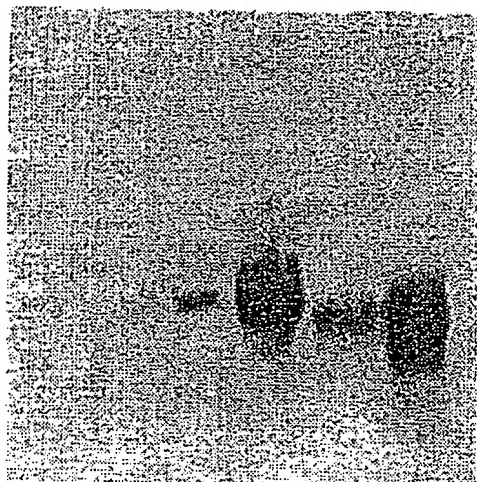
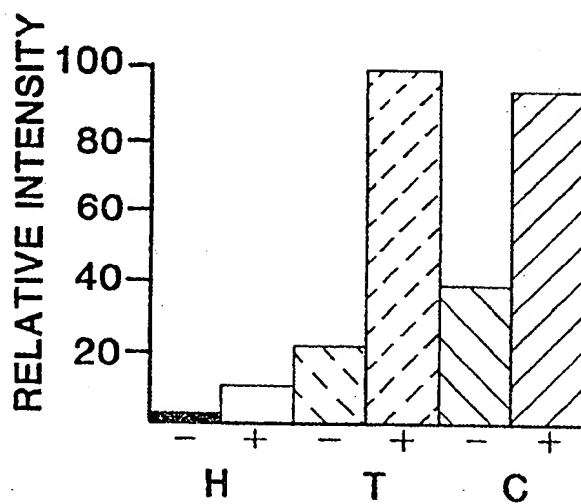
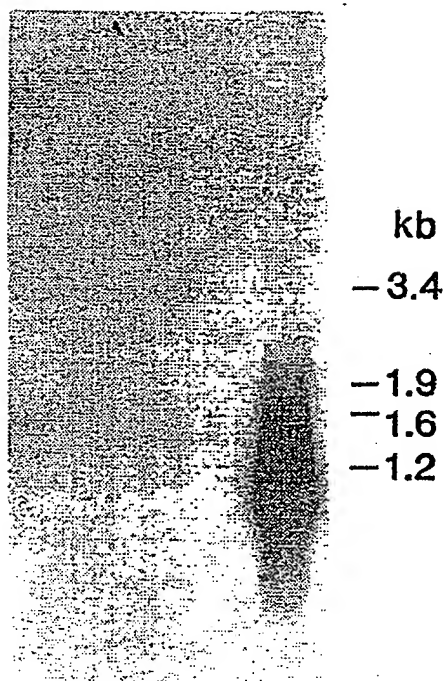


FIG. 3B

FIG. 3C



R Ca Co L E



Western blot analysis showing protein expression in various tissues. The blot is probed with anti- $\alpha$ -tubulin antibody. Molecular weight markers are indicated on the left: 43 kD, 30 kD, and 20 kD. The lanes are labeled A, B, C, D, E, F, G, and H at the bottom. An arrow points to a band at approximately 30 kD in lane H.

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 93/00141

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/82; C12N5/10;	C12N15/29; A01H5/00	C12N15/62; C12P21/02
<b>II. FIELDS SEARCHED</b>		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C12P ; A01H	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>		
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EMBL Nucleic Acid Sequence Database Accession No. X62353 20-2-1992 Release 31 A. thaliana gene for oleosin ----	12, 13, 26, 27
P, X	PLANT MOLECULAR BIOLOGY. vol. 18, no. 6, April 1992, DORDRECHT, THE NETHERLANDS. pages 1177 - 1179 VAN ROOIJEN, G.J.H. 'Nucleotide sequence of an Arabidopsis thaliana oleosin gene' see the whole document ----- -/--	12, 13, 26, 27
<sup>10</sup> Special categories of cited documents : <sup>"A"</sup> document defining the general state of the art which is not considered to be of particular relevance <sup>"E"</sup> earlier document but published on or after the international filing date <sup>"L"</sup> document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) <sup>"O"</sup> document referring to an oral disclosure, use, exhibition or other means <sup>"P"</sup> document published prior to the international filing date but later than the priority date claimed <sup>"T"</sup> later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention <sup>"X"</sup> document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step <sup>"Y"</sup> document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art <sup>"A"</sup> document member of the same patent family		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
05 AUGUST 1993	24 -08- 1993	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	MADDOX A.D.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category <sup>o</sup>	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PLANT PHYSIOLOGY. vol. 96, 1991, ROCKVILLE, MD, USA. pages 1395 - 1397 LEE, K., ET AL. 'Genomic nucleotide sequence of a Brassica napus 20-kilodalton oleosin gene'	12, 13
Y	see the whole document	1-11, 14-25, 28-30
Y	--- BIOLOGICAL CHEMISTRY HOPPE-SEYLER vol. 372, August 1991, page 537 MURPHY, D.J., ET AL. 'Molecular and biochemical mechanisms of storage oil-body formation and mobilisation in oilseeds' see abstract	1-11, 14-25, 28-30
Y	--- THE PLANT CELL. vol. 1, no. 10, 1989, ROCKVILLE, MD, USA. pages 969 - 976 MARCOTTE, W.R., ET AL. 'Absciscic acid-responsive sequences from the Em gene of wheat' see the whole document	1-11, 14-25, 28-30
A	--- PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 88, July 1991, WASHINGTON US pages 6181 - 6185 LEE, W.S., ET AL. 'Maize oleosin is correctly targeted to seed oil bodies in Brassic napus transformed with the maize oleosin gene' see the whole document	1-32
A	--- PLANT PHYSIOLOGY. vol. 97, 1991, ROCKVILLE, MD, USA. pages 1051 - 1058 HOLBROOK, LA., ET AL. 'Oilbody proteins in microspore-derived embryos of Brassica napus' see the whole document	1-30
A	--- J. EXP. BOT., SUPPL., MEETING HELD APRIL 7-12, 1991. vol. 42, no. 238, 1991, page 47 BATCHELDER, C., ET AL. 'Molecular genetics of oil body membrane proteins in Brassica napus and Arabidopsis' see abstract P8.58	1-32
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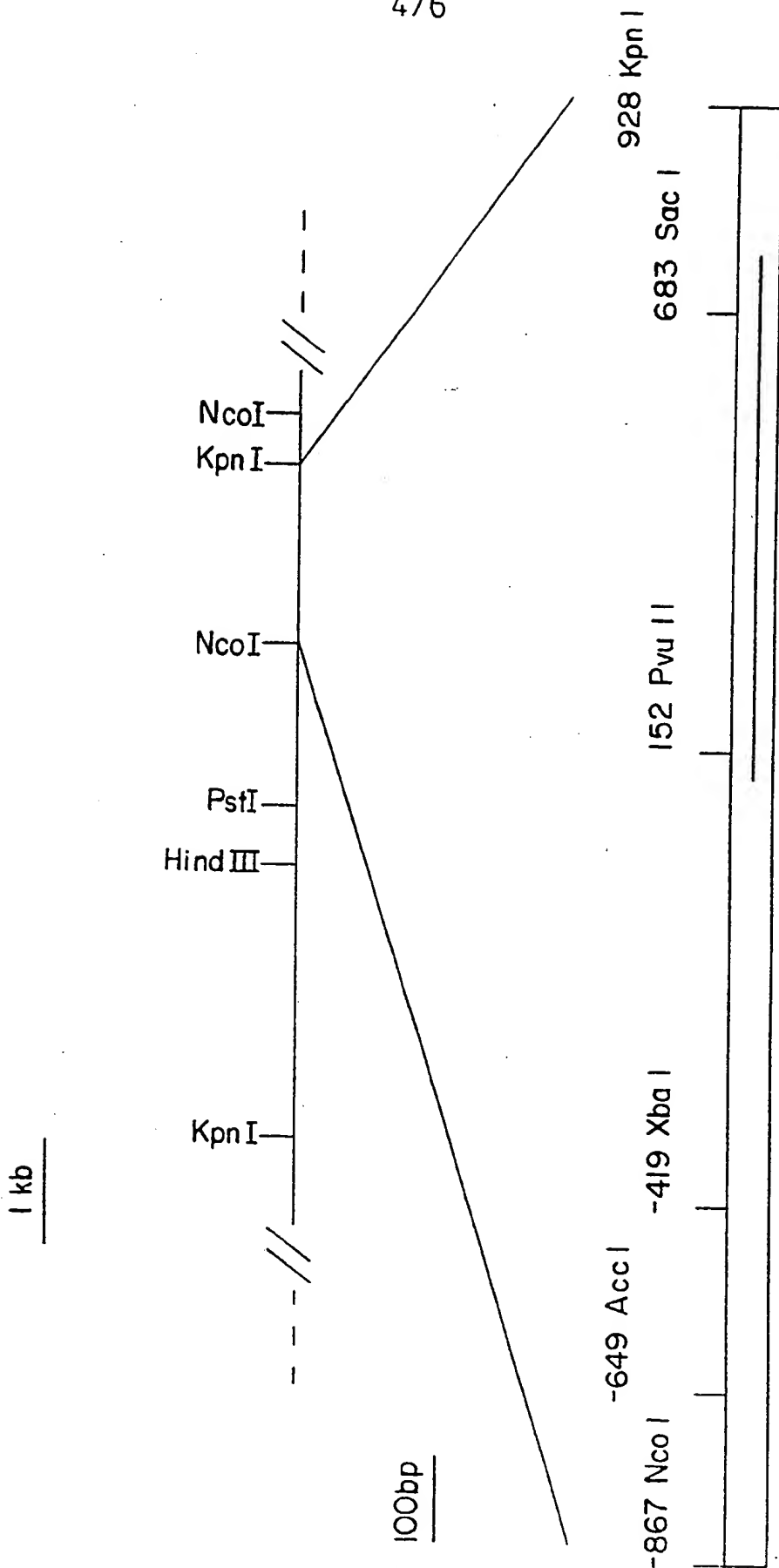


## III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	BIOTECHNOLOGY vol. 7, no. 9, September 1989, NEW YORK US pages 929 - 932 VANDEKERCKHOVE, J., ET AL. 'Enkephalins produced in transgenic plants using modified 2S seed storage proteins' see the whole document ----	31, 32
A	PLANT MOLECULAR BIOLOGY. vol. 15, 1990, DORDRECHT, THE NETHERLANDS. pages 905 - 912 YAMAGUCHI-SHINOZAKI, K., ET AL. 'Analysis of an ABA-responsive rice gene promoter in transgenic tobacco' see page 911, left column ----	2
A	PLANT MOLECULAR BIOLOGY. vol. 17, 1991, DORDRECHT, THE NETHERLANDS. pages 985 - 993 VILARDELL, J., ET AL. 'Regulation of the maize rab17 gene promoter in transgenic heterologous systems' see figure 2B -----	2

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1800 BASE PAIRS

FIG. 2B

FIG. 3A

H		T		C	
-	+	-	+	-	+

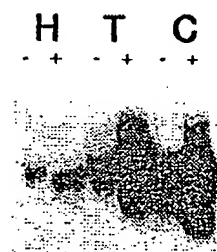
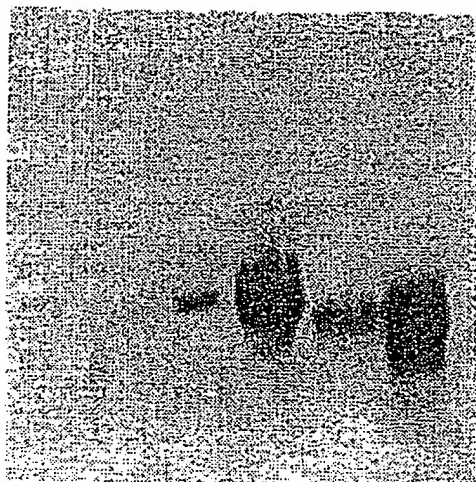
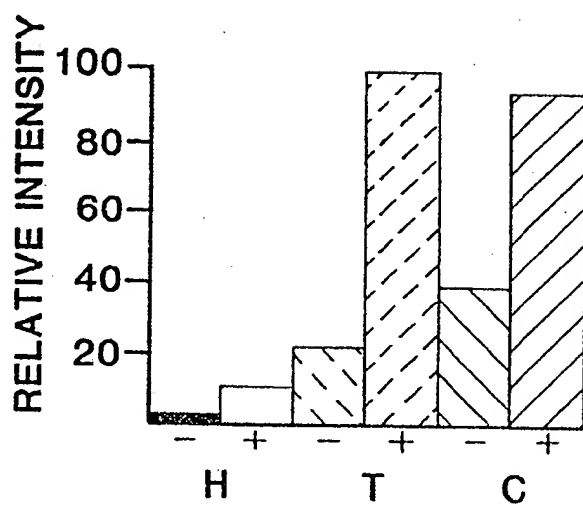


FIG. 3B

FIG. 3C



R Ca Co L E

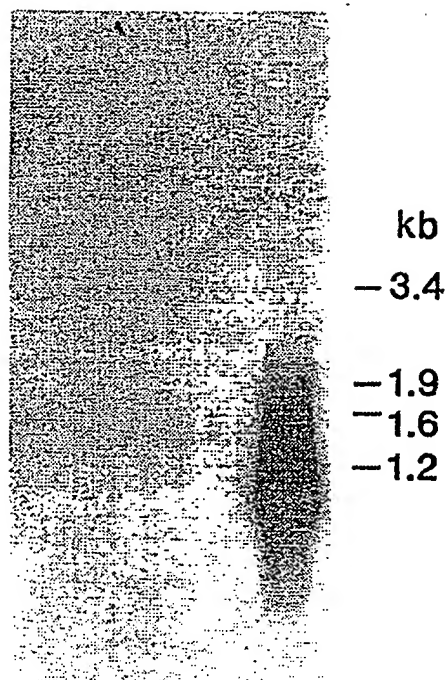
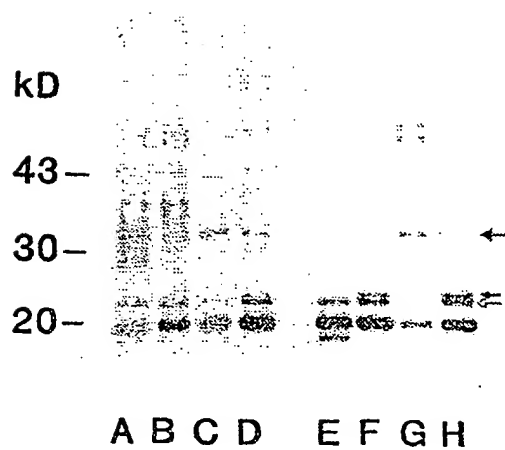


FIG. 5



## INTERNATIONAL SEARCH REPORT

International Application No

PCT/CA 93/00141

**I. CLASSIFICATION OF SUBJECT MATTER** (If several classification symbols apply, indicate all)<sup>6</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/29; C12N15/62; C12P21/02  
 C12N5/10; A01H5/00

**II. FIELDS SEARCHED**Minimum Documentation Searched<sup>7</sup>

Classification System

Classification Symbols

Int.Cl. 5 C12N ; C12P ; A01H

Documentation Searched other than Minimum Documentation  
 to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>

**III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>**

Category <sup>9</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
X	EMBL Nucleic Acid Sequence Database Accession No. X62353 20-2-1992 Release 31 A. thaliana gene for oleosin ---	12, 13, 26, 27
P, X	PLANT MOLECULAR BIOLOGY. vol. 18, no. 6, April 1992, DORDRECHT, THE NETHERLANDS. pages 1177 - 1179 VAN ROOIJEN, G.J.H. 'Nucleotide sequence of an Arabidopsis thaliana oleosin gene' see the whole document --- -/--	12, 13, 26, 27

<sup>9</sup> Special categories of cited documents: <sup>10</sup>

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"A" document member of the same patent family

**IV. CERTIFICATION**

Date of the Actual Completion of the International Search 05 AUGUST 1993	Date of Mailing of this International Search Report 24 -08- 1993
International Searching Authority EUROPEAN PATENT OFFICE	Signature of Authorized Officer MADDOX A.D.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	PLANT PHYSIOLOGY. vol. 96, 1991, ROCKVILLE, MD, USA. pages 1395 - 1397 LEE, K., ET AL. 'Genomic nucleotide sequence of a Brassica napus 20-kilodalton oleosin gene'	12, 13
Y	see the whole document	1-11, 14-25, 28-30
Y	--- BIOLOGICAL CHEMISTRY HOPPE-SEYLER vol. 372, August 1991, page 537 MURPHY, D.J., ET AL. 'Molecular and biochemical mechanisms of storage oil-body formation and mobilisation in oilseeds' see abstract	1-11, 14-25, 28-30
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A	--- PLANT PHYSIOLOGY. vol. 97, 1991, ROCKVILLE, MD, USA. pages 1051 - 1058 HOLBROOK, LA., ET AL. 'Oilbody proteins in microspore-derived embryos of Brassica napus' see the whole document	1-30
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A	PLANT MOLECULAR BIOLOGY. vol. 17, 1991, DORDRECHT, THE NETHERLANDS. pages 985 - 993 VILARDELL, J., ET AL. 'Regulation of the maize rab17 gene promoter in transgenic heterologous systems' see figure 2B -----	2

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